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# Platelet and leukocyte ROS production and lipoperoxidation are associated with high platelet reactivity in Non-ST elevation myocardial infarction (NSTEMI) patients on dual antiplatelet treatment

Matteo Becatti<sup>a,b,1</sup>, Claudia Fiorillo<sup>b,\*,1</sup>, Anna Maria Gori<sup>a</sup>, Rossella Marcucci<sup>a</sup>, Rita Panicia<sup>a</sup>, Betti Giusti<sup>a</sup>, Francesco Violi<sup>c</sup>, Pasquale Pignatelli<sup>c</sup>, Gian Franco Gensini<sup>a</sup>, Rosanna Abbate<sup>a</sup>

<sup>a</sup> Department of Experimental and Clinical Medicine, University of Florence, Italy

<sup>b</sup> Department of Biomedical, Experimental and Clinical Sciences, University of Florence, Florence, Italy

<sup>c</sup> Prima Clinica Medica, Sapienza-University of Rome, Rome, Italy

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## ABSTRACT

**Introduction:** High platelet reactivity (HPR) on dual antiplatelet therapy is a risk factor for adverse vascular events in acute coronary syndrome (ACS) patients. Several studies have shown that reactive oxygen species (ROS) may be involved in modulating platelet function.

**Methods:** In Non-ST elevation myocardial infarction (NSTEMI) patients ( $n = 132$ ) undergoing percutaneous coronary intervention (PCI) on dual antiplatelet therapy blood samples were collected within 24 h from 600 mg clopidogrel loading dose.

Platelet reactivity was assessed by light transmission aggregometry using 10  $\mu$ M ADP, 1 mM arachidonic acid (AA) and 2  $\mu$ g/ml collagen. ROS production and lipoperoxidation of circulating cells were determined, by FACSCanto flow cytometry. In these patients, we investigated: 1) the relationship between the amount of cellular ROS production/lipoperoxidation and platelet reactivity; 2) the association of cellular ROS production with the presence of high platelet reactivity to ADP and arachidonic acid (AA).

**Results:** Significantly higher levels of platelet and leukocyte-derived ROS were detected in 49 dual HPR (with platelet aggregation by AA  $\geq 20\%$  and by ADP  $\geq 70\%$ ) compared to non-HPR patients ( $n = 49$ ) [Platelet-derived ROS: +142%; Leukocyte-derived ROS: +14%,  $p < 0.0001$ ]. Similarly, dual HPR patients had significantly higher platelet and leukocyte lipoperoxidation than non-HPR patients [Platelet lipoperoxidation: +131%; Leukocyte lipoperoxidation: +14%,  $p < 0.001$ ].

After adjustment for several potential confounders, platelet-, leukocyte-derived ROS and platelet and leukocyte lipoperoxidation remained significantly associated to dual HPR.

The significant predictors of ADP, AA, and collagen platelet aggregation at multiple linear regression analysis, after adjusting for age, cardiovascular risk factors, procedural parameter, medications, leukocyte number and MPV, were platelet-, leukocyte-derived ROS and platelet and leukocyte lipoperoxidation ( $p < 0.01$ ).

**Conclusions:** Our results demonstrate that in NSTEMI patients on dual antiplatelet therapy, ROS production by and lipoperoxidation of platelets are strictly correlated to the different pathways of platelet aggregation and that ROS production and lipoperoxidation of platelets and leukocytes are predictors of non responsiveness to dual antiplatelet treatment.

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\* Corresponding author. Department of Biomedical, Experimental and Clinical Sciences, University of Florence, 50134 Florence, Italy. Tel.: +39 055 7949420; fax: +39 055 7949418.

E-mail address: [claudia.fiorillo@unifi.it](mailto:claudia.fiorillo@unifi.it) (C. Fiorillo).

<sup>1</sup> Both Authors equally contributed to the paper.

## 1. Introduction

High platelet reactivity (HPR) on dual antiplatelet therapy is an emerging prognostic factor which has been associated with adverse vascular events in the setting of acute coronary syndrome (ACS) patients undergoing percutaneous coronary intervention (PCI) and stenting [1–4].

The presence of the pro-thrombotic phenotype HPR may be explained by different clinical, and pharmacogenetic mechanisms mediated by biohumoral and cellular factors. Platelets are involved in inflammation, thrombosis and atherogenesis [5–9]. Monocytes, which are recruited to vessel walls, also play an important role in the acute phase of atherothrombosis. The interplay by platelets and other cells is evident in the trans-cellular metabolism of arachidonic acid, which promotes production of pro-inflammatory and vasoconstrictive compounds [e.g. leukotrienes and thromboxane (Tx)A<sub>2</sub>], and generates lipoxins, which are involved in the resolution of inflammation [10].

Although platelets display a prominent role in leukocyte activation, the reverse is also true. Superoxide anion, PAF, neutrophil elastase (NE) and cathepsin G (CG), all released from leukocytes, can induce platelet aggregation and secretion. Conversely, NO and ADPase released from unstimulated or weakly activated leukocytes attenuate platelet aggregation [11].

Experimental studies have shown that platelet aggregation is associated with considerable reactive oxygen species (ROS) production manifested as a burst of oxygen consumption [12], isoprostane formation [13–16] and an increase in GSSG [17]. This increase in GSSG arises, in part, from ROS such as O<sub>2</sub><sup>•</sup> [18,19] and H<sub>2</sub>O<sub>2</sub> [20] that are produced during the aggregation. Implicated sources for these ROS produced during aggregation include cyclooxygenase, lipoxygenase [21], and even NADPH oxidase [22,23].

In addition, different cell types, such as vascular smooth muscle cells, endothelial cells and fibroblasts, express a number of ROS-generating enzymes [24]. Besides their exposure to vascular wall-derived ROS, platelets are also exposed to high levels of ROS during the phagocytic burst reaction, which occurs during inflammation [25–27].

ACS is typically associated with increased ROS production, which may be responsible for a vicious circle (*a positive feedback*): proinflammatory cytokines trigger ROS production and ROS induce expression of pro-inflammatory cytokines [24]. However, the interplay between such burst of ROS and the entity of platelet activation is essentially speculative.

The aims of the present study were to investigate, in NSTEMI patients: 1) the relationship between the amount of ROS production by and lipoperoxidation of cellular elements involved in ACS and platelet activation through different pathways; 2) the association of cellular ROS production with the presence of high platelet reactivity to ADP and arachidonic acid (AA) stimulation in patients receiving both clopidogrel and acetyl salicylic acid (ASA) therapy.

Additional studies were performed to assess whether ROS production correlates with platelet function in the presence of isolated non responsiveness to clopidogrel or to ASA.

## 2. Materials and methods

### 2.1. Study population

In the framework of an ongoing project aimed to investigate the prevalence and the clinical implications of HPR in patients with ACS undergoing PCI on dual antiplatelet therapy, we consecutively investigated 49 NSTEMI patients with HPR by AA and ADP (dual HPR) and 49 without HPR (non-HPR). We also studied 17 NSTEMI patients with isolated HPR by AA and 17 with isolated HPR by ADP.

### 2.2. High on-treatment platelet reactivity (HPR)

For selecting study population we defined patients with dual HPR those patients with platelet aggregation induced by AA  $\geq$  20% and by ADP  $\geq$  70% according to the literature [28] and to studies from our group [1,29]. Isolated HPR by AA was defined in the

presence of platelet aggregation induced by AA  $\geq$  20% and by ADP  $<$  70%. Isolated HPR by ADP was defined in the presence of platelet aggregation induced by ADP  $\geq$  70% and by AA  $<$  20%.

All patients undergoing primary PCI received a clopidogrel loading dose (600 mg) followed by a daily dose of 75 mg. All patients received unfractionated heparin 70 IU/kg during the procedure and acetyl salicylic acid i.v. 500 mg followed by a daily dose of 100 mg by oral route.

Venous blood samples anticoagulated with 0.109 M sodium citrate (ratio 9:1) were taken from each patient within 24 h from 600 mg clopidogrel loading.

Patients were considered to have hypertension if they had been diagnosed as hypertensives according to the guidelines of European Society of Hypertension/European Society of Cardiology [30] or were taking antihypertensive drugs. Dyslipidemia was defined according to the third report of the National Cholesterol Education Program (NCEP III) [31] and diabetes in agreement with the American Diabetes Association [32].

The exclusion criteria included history of bleeding diathesis, platelet count  $\leq$  100,000/mm<sup>3</sup>, hematocrit  $\leq$  30%, creatinine  $\geq$  4.0 mg/dL, and glycoprotein (Gp) IIb/IIIa inhibitors use.

Informed written consent was obtained from all patients and the study was approved by the local Ethical Review Board. The investigation conforms with the principles outlined in the Declaration of Helsinki [33].

### 2.3. Platelet reactivity assessment

Turbidimetric platelet aggregation was used to measure agonist induced platelet aggregation. Whole blood samples were centrifuged for 10 min (min) at 250 g to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained on the remaining specimen by further centrifugation at 3000 g for 3 min. A platelet count was measured on the PRP and was adjusted to between  $180 \times 10^3/\mu\text{L}$  and  $300 \times 10^3/\mu\text{L}$  with PPP. PRP was stimulated with 10  $\mu\text{M}$  ADP (Mascia Brunelli, Milan, Italy), with 1 mmol arachidonic acid (AA) (Sigma–Aldrich, Milan, Italy) and with 2  $\mu\text{g}/\text{mL}$  collagen (Mascia Brunelli, Milan Italy) using a ATRACT 4 aggregometer (Helena Laboratories Italia S.p.A, Milan, Italy). Platelet aggregation (PA) according to Born's method was evaluated considering the maximal percentage of platelet aggregation in response to different stimuli (ADP-PA and AA-PA) after 10 min.

### 2.4. Assessment of ROS generation and lipoperoxidation by flow cytometry

#### 2.4.1. Sample preparation

After collection, 100  $\mu\text{L}$  EDTA-anticoagulated blood samples was stained for platelet surface antigens using 10  $\mu\text{L}$  Peridinin-chlorophyll-protein complex (PerCP)–conjugated human anti-CD61 monoclonal antibody and for leukocytes using 10  $\mu\text{L}$  PE-Cy7 Mouse Anti-Human CD45 (BD Pharmingen™, San Jose, CA, USA). The tubes were gently mixed and incubated at RT in the dark for 15 min. Surface-stained cells were resuspended in 2 mL of BD FACS Lysing Solution (BD Pharmingen™, San Jose, CA, USA), gently mixed, and incubated at RT in the dark for 10 min, following manufacturer's protocol. Subsequently, cells were centrifuged, the supernatant discarded and cells washed twice in PBS.

The level of intracellular ROS generation was determined after incubation with H<sub>2</sub>DCFDA (2.5  $\mu\text{M}$ ) (Invitrogen, CA, USA) in RPMI without serum and phenol red for 15 min at 37 °C [34–36].

As for lipoperoxidation, cells were incubated with BODIPY 581/591C11 (5  $\mu\text{M}$ ) (Invitrogen, CA, USA) in RPMI without serum and phenol red for 15 min at 37 °C [37,38].

2.4.2. FACS analysis

After labeling, cells were washed and resuspended in PBS and analyzed immediately using a FACSCanto flow cytometer (Becton–Dickinson, San Jose, CA). The sample flow rate was adjusted to about 1000 cells/s. For a single analysis, the fluorescence properties of 200,000 leukocytes were collected. The respective gates were defined using the distinctive forward-scatter and side-scatter properties of the individual cell populations. To avoid the inclusion of debris only CD61<sup>+</sup> platelets were analyzed. Moreover, cell viability was controlled by flow cytometry with propidium iodide staining and was found to exceed 95%. Platelet-leukocyte complexes (%) were determined based on the percentage of CD61-positive cells in the granulocyte, monocyte and lymphocyte population.

Data was analyzed using BD FACSDiva software (Becton–Dickinson, San Jose, CA, USA).

FACS gating strategies and representative FACS plots are shown in Fig. 1.

2.5. Statistical analysis

Statistical analysis was performed using the SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) software for Windows (Version 19.0). Values are presented as median and interquartile range.

The Mann–Whitney test for unpaired data was used for comparison between HPR and no-HPR patients groups. A correlation analysis for non-parametric data was performed to establish relationships between ROS generation, lipoperoxidation markers and agonist-induced platelet aggregation.

To examine the extent of cardiovascular risk factors (sex, familiar history of CAD, smoking habit, hypertension, diabetes, dyslipidemia, overweight), procedural characteristics (number of vessel disease, number of stents, ejection fraction (EF) ≤ 40%) and

pharmacological treatment (ACE-inhibitors, Statins and proton pump inhibitors and Beta Blockers) in influencing HPR we used Chi-square test.

To evaluate the independent predictors of dual HPR we used a multivariable logistic regression analysis adjusted for the variables that at univariate analysis had a *p* value <0.10 (age, leukocyte count, mean platelet volume-MPV and ejection fraction-EF < 40%), plus cell-derived ROS and cell lipoperoxidation. Cell-derived ROS production and lipoperoxidation variables were transformed into square root values, due to the skewed distribution of the variables.

We used linear regression models to test the independent associations of AA- or ADP-induced platelet aggregation with platelet and leukocytes-derived ROS and platelet and leukocytes lipoperoxidation. We also evaluated the independent association of AA- or ADP-induced platelet aggregation with cardiovascular risk factors (age, sex, familiar history of CAD, smoking habit, hypertension, diabetes, dyslipidemia, overweight), leukocyte count, mean platelet volume (MPV), procedural characteristics (number of vessel disease, number of stents and EF < 40%) and pharmacological treatment (ACE-inhibitors, Statins and proton pump inhibitors and Beta Blockers) as potential predictors. In the final model of multivariate linear regression analysis we added each oxidative stress parameters (separately due to the collinearity of these factors) to the variables that at univariate analysis had a *p* value < 0.10 (age, leukocyte count, mean platelet volume-MPV and ejection fraction-EF < 40%). Results are expressed as  $\beta \pm SE$  *P* < 0.05 was considered to be statistically significant.

3. Results

Clinical, demographic, and laboratory characteristics of patients according to the presence of dual HPR are shown in Table 1 and Fig. 2.

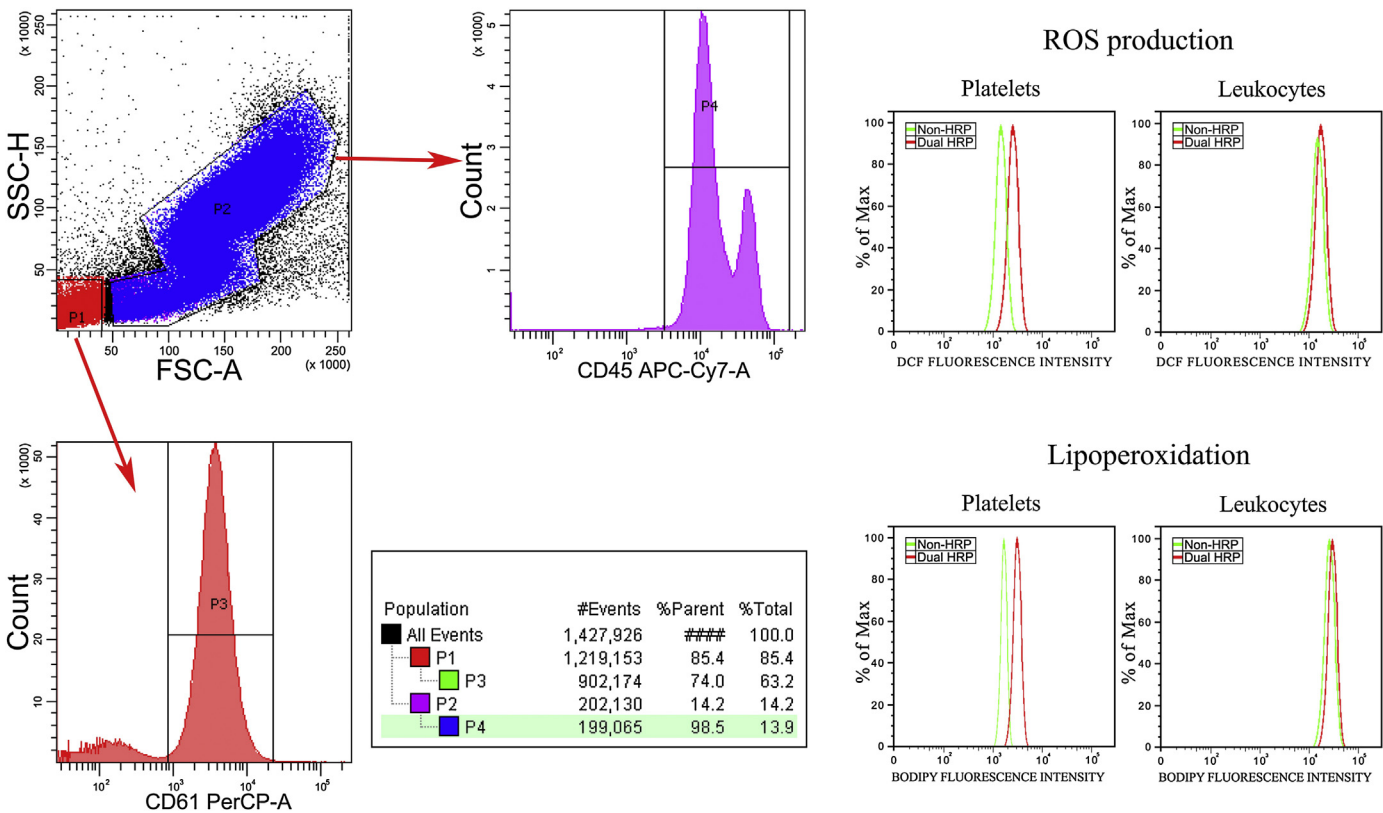


Fig. 1. FACS gating strategies and representative FACS plots.



**Table 1**

Clinical characteristics of acute coronary syndrome patients with and without dual high platelet reactivity (dual HPR).

	Non-HPR (n = 49)	Dual HPR (n = 49)	P dual HPR vs non-HPR
Males/Females	40/9	32/17	0.067
Age (median, range)	67 (42–90)	78 (55–91)	0.001
Hypertension (n,%)	27 (55.1)	31 (63.2)	0.411
Smoking habit (n,%)	24 (48.9)	16 (32.6)	0.100
Dyslipidemia (n,%)	22 (44.9)	21 (42.9)	0.839
Diabetes (n,%)	10 (20.4)	11 (22.4)	0.806
Family history of CAD (n,%)	6 (12.2)	5 (10.2)	0.749
Overweight (n,%)	5 (10.2)	6 (12.2)	0.749
1-vessel disease	30 (61.2)	33 (67.4)	0.673
2-vessel disease	11 (22.5)	10 (20.4)	0.999
3-vessel disease	8 (16.3)	6 (12.2)	0.774
Number of stents	1.45 ± 1.1	1.47 ± 1.3	0.505
ACE-inhibitors	30 (61.2)	31 (63.3)	0.999
Statins	31 (63.3)	30 (61.2)	0.999
Proton Pump Inhibitors*	44 (89.8)	45 (91.8)	0.999
Beta Blockers	21 (42.9)	20 (40.8)	0.999
AA-platelet aggregation (%)	15 (8–19)	31 (20–100)	<0.0001
ADP-platelet aggregation (%)	46 (8–69)	77 (70–100)	<0.0001
Collagen-platelet aggregation (%)	23 (4–66)	60 (30–100)	<0.0001
Leukocytes (×10 <sup>9</sup> /L)	6.74 (3.73–14.41)	7.87 (3.98–15.8)	0.044
Platelets (×10 <sup>9</sup> /L)	207 (142–463)	197 (144–413)	0.704
MPV (fL)	10.9 (8.9–12.5)	11.2 (9.2–13.0)	0.073

CAD = coronary artery disease; Overweight was defined in the presence of BMI >25, mean platelet volume (MPV).

### 3.1. ROS generation and lipoperoxidation

In Fig. 2 we reported median values (interquartile range) according to the presence of dual HPR: significantly higher levels of platelet and leukocyte-derived ROS were detected in dual HPR with respect to non-HPR patients. Similarly, platelet and leukocyte lipoperoxidation values were significantly higher in dual HPR with respect to non-HPR patients (Fig. 2). Platelet- and leukocyte-derived ROS and lipoperoxidation significantly correlated with AA-, ADP-, and collagen-induced platelet aggregation (Fig. 3).

We evaluated 17 patients with isolated HPR by AA and 17 patients with isolated HPR by ADP. The clinical and laboratory characteristics of the patients with isolated HPR by AA or by ADP were similar to those observed in patients with dual HPR (data not shown).

In patients with isolated HPR by AA and in those with isolated HPR by ADP platelet-derived ROS and platelet lipoperoxidation levels were significantly ( $p < 0.0001$ ) higher than in patients without HPR and lower than in patients with dual HPR (Fig. 4).

No significant difference in platelet ROS production and platelet lipoperoxidation levels between isolated HPR by AA and isolated HPR by ADP was found.

In dual HPR patients platelet–leukocyte complexes (%) in the granulocyte, monocyte and lymphocyte population were significantly increased ( $p < 0.01$ ) compared to non-HPR patients ( $19.5 \pm 5.7$  vs  $11.8 \pm 4.3$ ;  $26.0 \pm 4.8$  vs  $18.7 \pm 3.9$ ;  $17.4 \pm 3.6$  vs  $10.6 \pm 1.8$  respectively).

### 3.2. Correlation between parameters investigated

Platelet-derived ROS significantly correlated with leukocyte-derived ROS ( $r = 0.50$ ,  $p < 0.0001$ ), as well as with platelet, and leukocyte lipoperoxidation ( $r = 0.82$  and  $r = 0.51$ ,  $p < 0.0001$ ).

Among clinical characteristics, age was significantly correlated with platelet-derived ROS ( $r = 0.32$ ,  $p < 0.001$ ) and with platelet and leukocyte lipoperoxidation ( $r = 0.35$ ,  $p < 0.001$ ;  $r = 0.25$ ,  $p = 0.007$ ). No correlation between platelet function, ROS production or lipoperoxidation and platelet volume was found.

### 3.3. Regression analyses

The significant predictors of dual HPR were shown in Table 2.

After adjustment for several potential confounders, age, EF < 40%, platelet-, leukocyte-derived ROS and platelet and leukocyte lipoperoxidation remained significantly associated to dual HPR (Fig. 5).

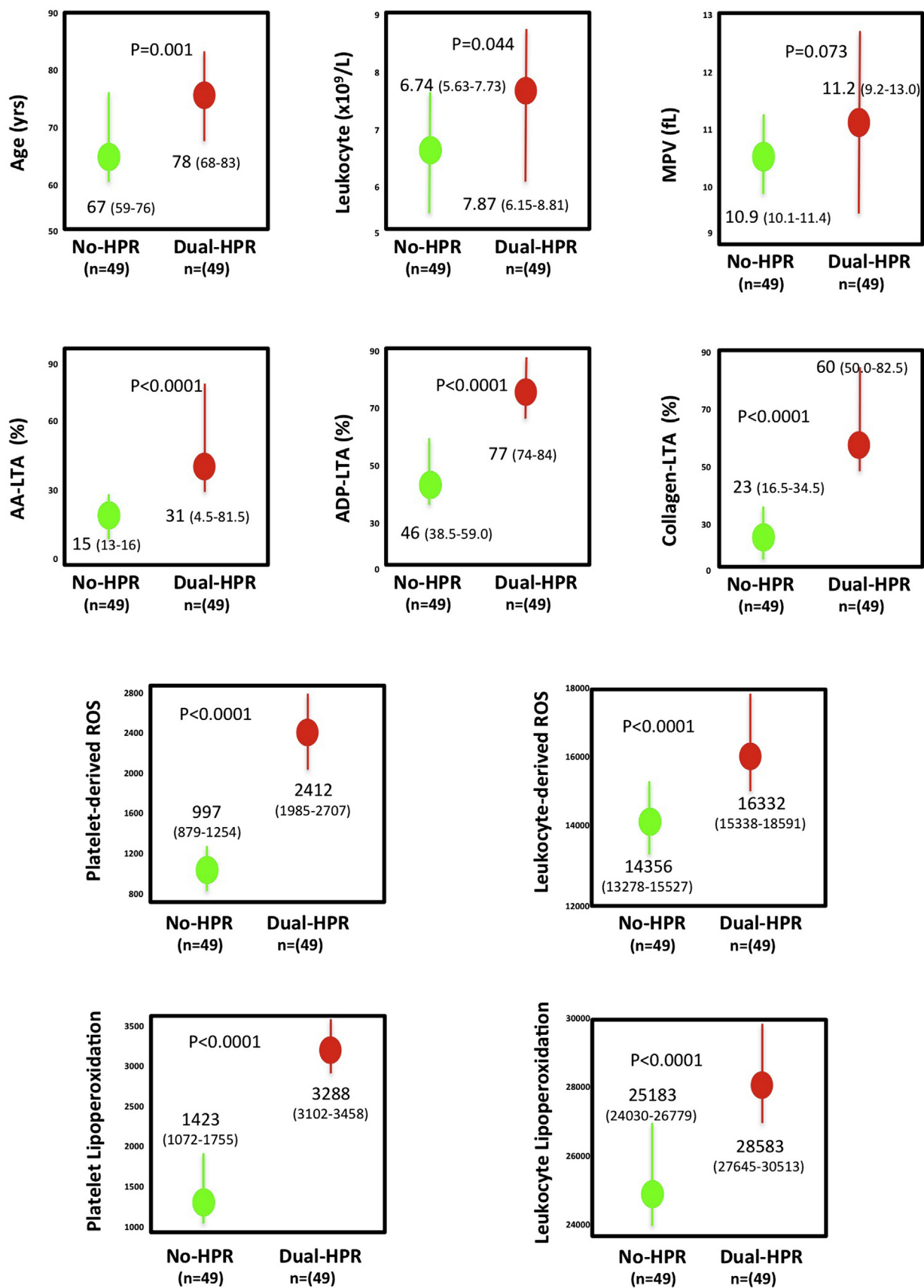
The significant predictors of ADP, AA, and collagen platelet aggregation at multiple linear regression analysis, after adjusting for age, leukocyte number, MPV and EF < 40% are reported in Table 3.

## 4. Discussion

This study provides the novel findings that in patients with ACS on dual antiplatelet therapy: 1) ROS production and lipoperoxidation by platelets and leukocytes are predictors of non-responsiveness to dual antiplatelet treatment; 2) ROS and lipoperoxidation by platelets and leukocytes are correlated to the different pathways of platelet aggregation 3) isolated clopidogrel as well as aspirin non-responsiveness are associated with an up-regulation of platelet and leukocytes ROS production and lipoperoxidation.

ROS are produced by blood cells including platelets and leukocytes and play a role in favoring platelet activation by several mechanisms. ROS may, in fact, inactivate or inhibit the biosynthesis of NO, a powerful antiaggregating and vasodilator molecule, or elicit formation of isoprostanes, which contribute to propagate aggregation via GpIIb/IIIa activation [14]. Previous studies demonstrated in vitro that, upon anoxia, platelets exhibit a burst of ROS which contributes to up-regulate COX1, so enhancing platelet TxA<sub>2</sub> formation [39]. This datum was corroborated in vivo by showing that after PCI, a typical model of ischemia-reperfusion, platelet TxA<sub>2</sub> formation was over-expressed consequently to enhanced ROS formation [40]. This finding prompted to hypothesize that a burst of ROS occurring after PCI may be responsible for platelet activation, but in vivo evidence supporting such interplay had not yet been investigated. To explore this issue we analyzed if platelet production of ROS could be implicated in the platelet non-responsiveness to antiplatelet drugs, observed after PCI [3,4] in ACS patients. In particular, the present study was designed to investigate the relationship between platelet oxidative stress and platelet function in patients with high residual platelet reactivity to both arachidonic acid and ADP, the so called dual non-responders. The potential clinical relevance of the study is outlined by previous studies [1,2] in which we found out that dual non-responsiveness in the acute phase of ACS is the most accurate predictor of patients at high risk of stent thrombosis and death.

The novelty of our study is in the detection of a higher production of ROS in patients who did not respond to dual antiplatelet treatment compared with those who did respond, suggesting a cause-effect relationship between the burst of ROS and impaired platelet inhibition. Consistent with this hypothesis was the direct correlation between oxidative stress, as measured by platelet ROS and lipoperoxidation, and responsiveness to several agonists, such as ADP, AA, and collagen, in patients on dual antiplatelet treatment undergoing PCI. The plausibility of this interpretation is based on previous data indicating that ROS serve to amplify platelet response to common agonist via release of ADP, inactivation of NO and/or production of isoprostanes [14]. In accordance with this suggestion,



**Fig. 2.** Platelet-, leukocyte-derived ROS and platelet and leukocyte lipoperoxidation according to the presence or the absence of dual high platelet reactivity (dual HPR) (platelet aggregation by AA  $\geq 20\%$  and by ADP  $\geq 70\%$ ).

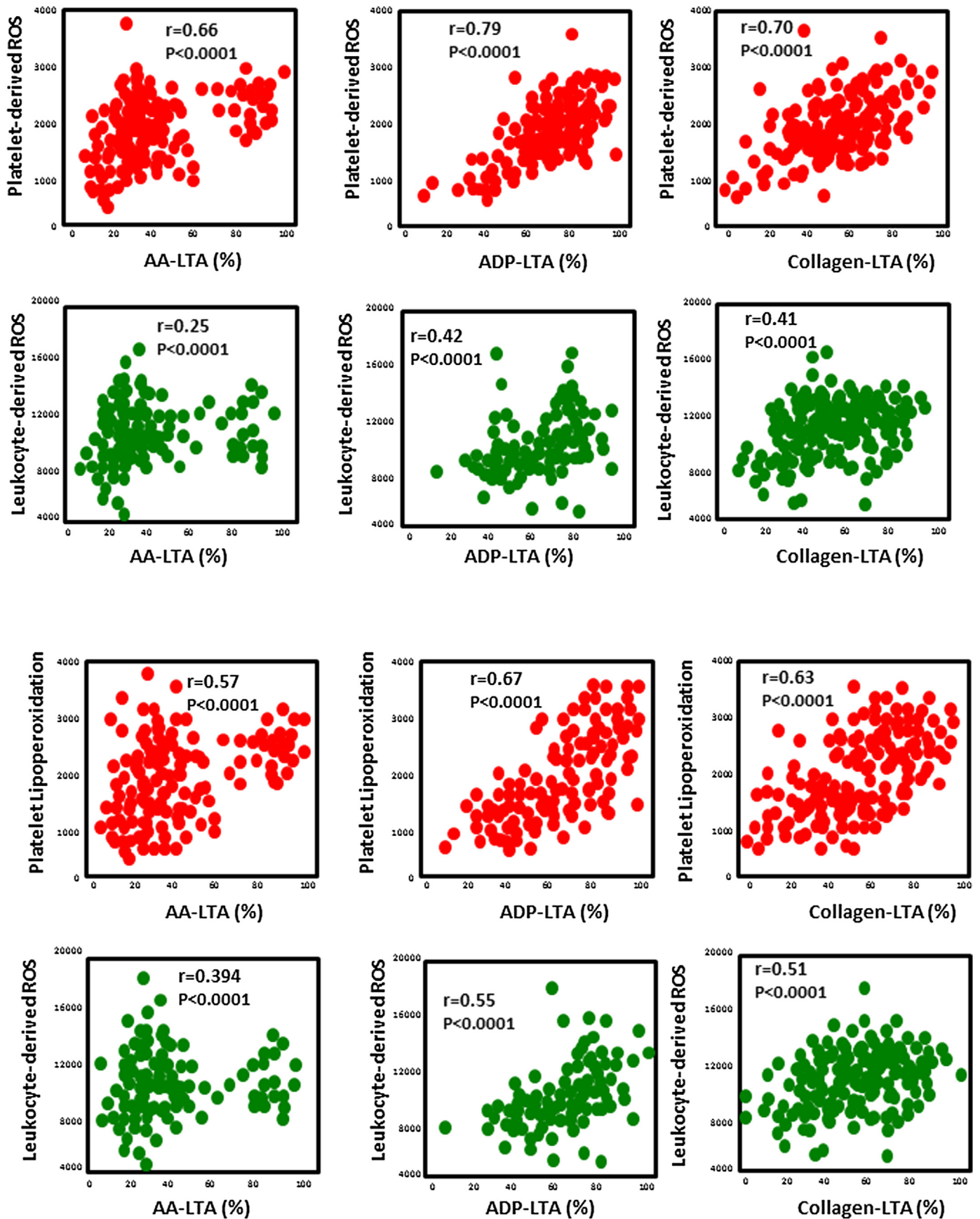


Fig. 3. Correlations between Platelet- and leukocyte-derived ROS and lipoperoxidation with AA-, ADP-, and collagen-induced platelet aggregation.

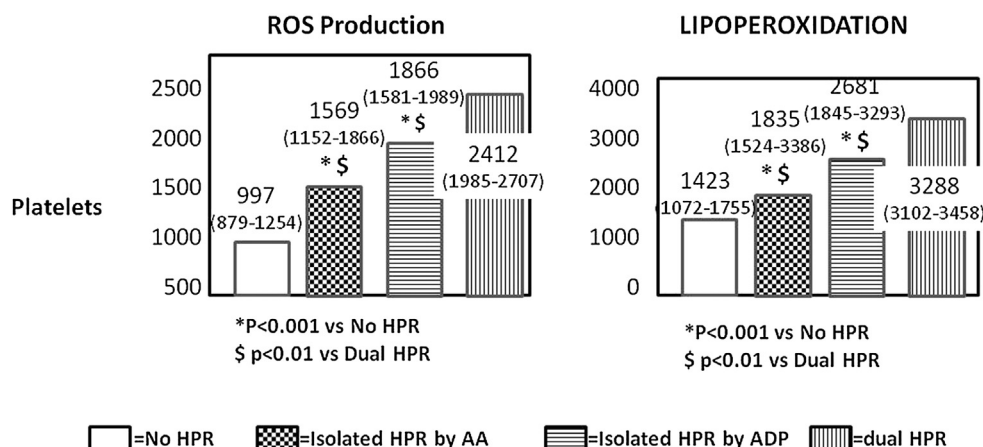


Fig. 4. ADP Platelet-derived ROS and platelet lipoperoxidation levels according to the presence of dual HPR, isolated HPR by AA and isolated HPR.

a direct correlation between platelet ROS and platelet function was detected also in patients with isolated non-responsiveness to clopidogrel or aspirin; in fact either P2Y<sub>12</sub> and AA pathways are associated with ROS formation and ultimately with activation of platelet function [37,14,40]. The correlation between leukocyte oxidative stress and platelet non-responsiveness to dual antiplatelet drugs is another novelty of the present study. This suggests that the burst of ROS after PCI in ACS patients occurs in platelets and to a lesser extent in leukocytes which, in turn, contribute to activate platelets with a ROS-mediated mechanism. This finding provides further insight into our previous results [40] claiming a link between inflammation and platelet hyper-function.

As suggested by several authors, the cross-talk between platelets and leukocytes plays a significant role in thrombosis and inflammation [1–4]. Increased formation of platelet–leukocyte complexes is clinically observed in acute coronary syndromes [6], myocardial infarction [7], and vascular diseases including arteriosclerosis [8] and inflammatory disorders [9].

The results of our study clearly show that in dual HPR patients platelet–leukocyte complexes (%) in the granulocyte, monocyte and lymphocyte population were significantly increased compared to non-HPR patients. This finding agrees with the enhanced platelet ROS production in dual HPR patients.

Table 2

Univariate logistic analysis for dual high platelet reactivity (dual HPR) HPR in ACS patients on dual antiplatelet treatment.

	OR (95% CI) univariate analysis	P value
Age (yrs)	1.07 (1.03–1.12)	0.002
Gender (Females vs Males)	2.07 (0.83–5.15)	0.117
Smoking habits	0.55 (0.24–1.24)	0.150
Hypertension	1.40 (0.63–3.15)	0.412
Diabetes	0.86 (0.35–2.11)	0.855
Dyslipidemia	0.78 (0.35–1.73)	0.543
History of CAD	0.81 (0.23–2.87)	0.749
Overweight (BMI > 25)	1.57 (0.41–5.95)	0.507
Multivessel PCI	1.07 (0.61–1.88)	0.823
Number of stent	0.96 (0.53–1.74)	0.893
Ejection fraction <40%	3.38 (0.85–13.34)	0.083
Leukocytes ( $\times 10^9/L$ )	1.21 (0.99–1.46)	0.060
Mean Platelet Volume (fL)	1.57 (0.98–2.53)	0.061
Platelet number ( $\times 10^9/L$ )	0.998 (0.99–1.04)	0.563
Platelet-induced ROS	1.86 (1.38–2.49)	<0.0001
Leukocyte-derived ROS	1.09 (1.04–1.12)	<0.0001
Platelet lipoperoxidation	1.62 (1.33–1.98)	<0.0001
Leukocyte lipoperoxidation	1.47 (1.28–1.69)	<0.0001

Odds ratio (OR) and 95% confidence interval (CI).

In the clinical setting of the present results, non-responsiveness to dual antiplatelet therapy cannot be ascribed to the reduced compliance as blood withdrawal for platelet function studies were obtained when patients were still in intensive coronary units. Nevertheless the study has limitations and implication which should be acknowledged. We cannot exclude that other mechanisms, related to pharmacogenetic, metabolic, drug interferences participated in determining non responsiveness; however, the clear-cut difference in platelet ROS production between responders and non-responders indicates that, whatever is the mechanism of platelet hyperactivity, ROS formation by platelets and leukocytes may represent a plausible mechanism accounting for platelet hyper-reactivity after PCI in ACS.

Our hypothesis on the role of ROS in impaired platelet responsiveness is based essentially on correlation analysis. Also, the mechanism accounting for platelet burst of ROS was not explored. Even if it was not specifically investigated in the present study, activation of NOX2 is likely to be implicated in ROS formation as NOX2 is a key enzyme of ROS production in both platelets and leukocytes [14,41–44]. Also, previous study from our group demonstrated that after PCI serum levels of NOX2, which maximally reflect NOX2 activation by platelets and leukocytes, was up-regulated and contributed to enhance serum TxB<sub>2</sub> [40].

Based on these findings, a cause–effect relationship between the burst of ROS by platelets and leukocytes and platelet hyper-

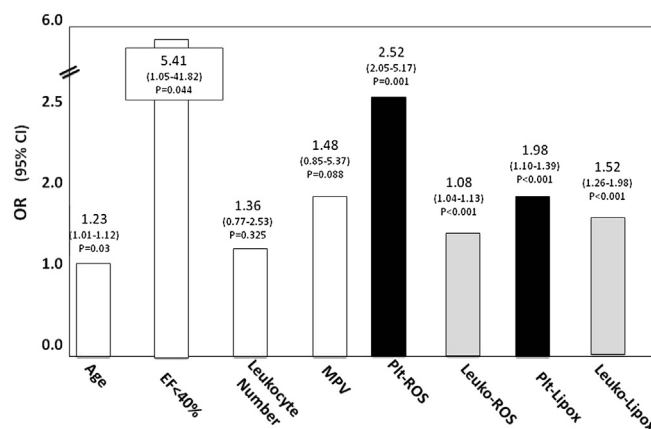


Fig. 5. Multivariable logistic regression analysis for dual HPR adjusted for age, sex, smoking habit, leukocyte count, mean platelet volume, plus cell-derived ROS and cell lipoperoxidation.



**Table 3**

The significant predictors of ADP-, AA-, and collagen-platelet aggregation at multiple linear regression analysis, after adjusting for age, leukocyte number, mean platelet volume and ejection fraction (EF) < 40%.

	ADP-platelet aggregation		AA-platelet aggregation		Collagen-platelet aggregation	
	Beta ± SE	p	Beta ± SE	p	Beta ± SE	p
Age	0.19 ± 0.08	0.036	0.21 ± 0.10	0.042	0.29 ± 0.08	0.01
Leukocyte number	0.09 ± 0.06	0.354	0.11 ± 0.08	0.230	0.09 ± 0.05	0.288
MPV	0.10 ± 0.08	0.096	0.09 ± 0.07	0.10	0.16 ± 0.09	0.066
EF < 40%	0.22 ± 0.09	0.01	0.15 ± 0.08	0.088	0.21 ± 0.08	0.01
Platelet-derived ROS	0.74 ± 0.03	0.0001	0.57 ± 0.03	0.0001	0.69 ± 0.03	0.0001
Leukocyte-derived ROS	0.32 ± 0.04	0.0001	0.39 ± 0.10	0.035	0.32 ± 0.02	0.002
Platelet lipoperoxidation	0.77 ± 0.03	0.0001	0.56 ± 0.68	0.0001	0.68 ± 0.03	0.0001
Leukocyte lipoperoxidation	0.52 ± 0.02	0.001	0.44 ± 0.03	0.0001	0.52 ± 0.02	0.0001

Beta ± standard error (SE).

activation observed after PCI in the acute phase of ACS cannot be drawn by the present study. Interventional trials with antioxidants which inhibit lipid peroxidation such as vitamin E or quench ROS such as vitamin C should be necessary to support our hypothesis. Of note, a previous study from our group demonstrated that 1 g vitamin C infusion during PCI has been able to down-regulate NOX2 activity and to reduce platelet TxA<sub>2</sub> formation [40].

In conclusion, this study provides the first evidence that in the acute phase of ACS after PCI platelets and leukocytes produce a burst of ROS, which may be implicated in platelet non-responsiveness to dual antiplatelet treatment. This finding provides a rationale to investigate if the use of antioxidants such as vitamin C may be a therapeutic tool to modulate platelet hyper-responsiveness detected after PCI in ACS.

## Disclosures

The Authors report no conflict of interest.

### What is known on this topic

High platelet reactivity on dual antiplatelet treatment is associated with increased risk of cardiovascular events in acute coronary syndromes.

Oxidative stress plays a role in the pathophysiology of cardiovascular diseases.

### What this paper adds

ROS production and lipoperoxidation by platelets and leukocytes are predictors of non-responsiveness to dual antiplatelet treatment.

This finding provides a rationale to investigate if the use of antioxidants such as vitamin C may be a therapeutic tool to modulate platelet hyper-responsiveness detected after PCI in ACS.

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## References

- [1] Parodi G, Marcucci R, Valenti R, et al. High residual platelet reactivity after clopidogrel loading and long-term cardiovascular events among patients with acute coronary syndromes undergoing PCI. *JAMA* 2011;306:1215–23.
- [2] Gori AM, Marcucci R, Migliorini A, et al. Incidence and clinical impact of dual nonresponsiveness to aspirin and clopidogrel in patients with drug-eluting stents. *J Am Coll Cardiol* 2008;52:734–9.
- [3] Marcucci R, Gori AM, Panicia R, et al. Cardiovascular death and nonfatal myocardial infarction in acute coronary syndrome patients receiving coronary stenting are predicted by residual platelet reactivity to ADP detected by a point-of-care assay: a 12-month follow-up. *Circulation* 2009;119:237–42.
- [4] Ferreira JL, Homs S, Berdejo J, et al. Clopidogrel pretreatment in primary percutaneous coronary intervention: prevalence of high on-treatment platelet reactivity and impact on preprocedural patency of the infarct-related artery. *Thromb Haemost* 2013;110(1).
- [5] Marcucci R, Gori AM, Panicia R, et al. Residual platelet reactivity is associated with clinical and laboratory characteristics in patients with ischemic heart disease undergoing PCI on dual antiplatelet therapy. *Atherosclerosis* 2007;195:e217–23.
- [6] Giusti B, Gori AM, Marcucci R, et al. Role of glycoprotein Ia gene polymorphisms in determining platelet function in myocardial infarction patients undergoing percutaneous coronary intervention on dual antiplatelet treatment. *Atherosclerosis* 2008;196:341–8.
- [7] Cecchi E, Marcucci R, Panicia R, et al. Effect of blood hematocrit and erythrocyte deformability on adenosine 5'-diphosphate platelet reactivity in patients with acute coronary syndromes on dual antiplatelet therapy. *Am J Cardiol* 2009;104:764–8.
- [8] Gori AM, Cesari F, Cinotti S, et al. The balance between pro- and anti-inflammatory cytokines is associated with platelet aggregability in acute coronary syndrome patients. *Atherosclerosis* 2009;202:255–62.
- [9] Marcucci R, Cesari F, Cinotti S, et al. ADAMTS-13 activity in the presence of elevated von Willebrand factor levels as a novel mechanism of residual platelet reactivity in high risk coronary patients on antiplatelet treatment. *Thromb Res* 2008;123:130–6.
- [10] Ferroni P, Vazzana N, Riondino S, Cuccurullo C, Guadagni F, Davì G. Platelet function in health and disease: from molecular mechanisms, redox considerations to novel therapeutic opportunities. *Antioxid Redox Signal* 2012;17:1447–85.
- [11] Salvemini D, de Nucci G, Gryglewski RJ, Vane JR. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc Natl Acad Sci U S A* 1989 Aug;86(16):6328–32.
- [12] Bressler NM, Broekman MJ, Marcus AJ. Concurrent studies of oxygen consumption and aggregation in stimulated human platelets. *Blood* 1979;53:167–78.
- [13] Krotz F, Sohn HY, Pohl U. Reactive oxygen species: players in the platelet game. *Arterioscler Thromb Vasc Biol* 2004;24:1988–96.
- [14] Pignatelli P, Carnevale R, Di Santo S, et al. Inherited human gp91phox deficiency is associated with impaired isoprostane formation and platelet dysfunction. *Arterioscler Thromb Vasc Biol* 2011;31:423–34.
- [15] Krötz F, Sohn HY, Gloe T, et al. NAD(P)H oxidase-dependent platelet superoxide anion release increases platelet recruitment. *Blood* 2002;100:917–24.
- [16] Violi F, Pignatelli P. Platelet oxidative stress and thrombosis. *Thromb Res* 2012;129:378–81.
- [17] Burch JW, Burch PT. Glutathione disulfide production during arachidonic acid oxygenation in human platelets. *Prostaglandins* 1990;39:123–34.

- [18] Freedman JE, Keaney Jr JF. Nitric oxide and superoxide detection in human platelets. *Methods Enzymol* 1999;301:61–70.
- [19] Marcus AJ, Silk ST, Safier LB, Ullman HL. Superoxide production and reducing activity in human platelets. *J Clin Invest* 1977;59:149–58.
- [20] Stuart MJ, Holmsen H. Hydrogen peroxide, an inhibitor of platelet function: effect on adenine nucleotide metabolism, and the release reaction. *Am J Hematol* 1977;2:53–63.
- [21] Singh D, Greenwald JE, Bianchini J, Metz EN, Sagone Jr AL. Evidence for the generation of hydroxyl radical during arachidonic acid metabolism by human platelets. *Am J Hematol* 1981;11:233–40.
- [22] Krotz F, Sohn HY, Gloe T, et al. NAD(P)H oxidase-dependent platelet superoxide anion release increases platelet recruitment. *Blood* 2002;100:917–24.
- [23] Seno T, Inoue N, Gao D, et al. Involvement of NADH/NADPH oxidase in human platelet ROS production. *Thromb Res* 2001;103:399–409.
- [24] Loscalzo J. Oxidant stress: a key determinant of atherothrombosis. *Biochem Soc Trans* 2003;31:1059–61.
- [25] Pratico D, Iuliano L, Alessandri C, et al. Polymorphonuclear leukocyte-derived O<sub>2</sub>-reactive species activate primed platelets in human whole blood. *Am J Physiol* 1993;264:1582–7.
- [26] Cerwinka WH, Cooper D, Kriegelstein CF, Ross CR, McCord JM, Granger DN. Superoxide mediates endotoxin-induced platelet-endothelial cell adhesion in intestinal venules. *Am J Physiol Heart Circ Physiol* 2003;284:535–41.
- [27] Watt J, Ewart MA, Greig FH, Oldroyd KG, Wadsworth RM, Kennedy S. The effect of reactive oxygen species on whole blood aggregation and the endothelial cell-platelet interaction in patients with coronary heart disease. *Thromb Res* 2012;130:210–5.
- [28] Gum PA, Kottke-Marchant K, Welsh PA, White J, Topol EJ. A prospective, blinded determination of the natural history of aspirin resistance among stable patients with cardiovascular disease. *J Am Coll Cardiol* 2003;41:961–5.
- [29] Buonamici P, Marcucci R, Migliorini A, et al. Impact of platelet reactivity after clopidogrel administration on drug-eluting stent thrombosis. *J Am Coll Cardiol* 2007;49:2312–7.
- [30] Practice guidelines for primary care physicians: 2003 ESH/ESC hypertension guidelines. ESH/ESC Hypertension Guidelines Committee. *Hypertension* 2003;21:1779–86.
- [31] Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adult (Adult Treatment Panel III) final report. *Circulation* 2002;106:3143–421.
- [32] Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 2003;26:5–20.
- [33] World Medical Association Declaration of Helsinki. Recommendations guiding physicians in biomedical research involving human subjects. *Cardiovasc Res* 1997;35:2–3.
- [34] Becatti M, Prignano F, Fiorillo C, et al. The involvement of Smac/DIABLO, p53, NF- $\kappa$ B, and MAPK pathways in apoptosis of keratinocytes from perilesional vitiligo skin: protective effects of curcumin and capsaicin. *Antioxid Redox Signal* 2010;13:1309–21.
- [35] Becatti M, Taddei N, Cecchi C, et al. SIRT1 modulates MAPK pathways in ischemic-reperfused cardiomyocytes. *Cell Mol Life Sci* 2012;69:2245–60.
- [36] Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol* 1983;130:1910–7.
- [37] Prignano F, Pescitelli L, Becatti M, et al. Ultrastructural and functional alterations of mitochondria in perilesional vitiligo skin. *J Dermatol Sci* 2009;54:157–67.
- [38] Drummen GP, van Liebergen LC, Op den Kamp JA, Post JA. C11-BODIPY(581/591), an oxidation-sensitive fluorescent lipid peroxidation probe: (micro) spectroscopic characterization and validation of methodology. *Free Radic Biol Med* 2002;33:473–90.
- [39] Leo R, Pratico D, Iuliano L, et al. Platelet activation by superoxide anion and hydroxyl radicals intrinsically generated by platelets that had undergone anoxia and then reoxygenated. *Circulation* 1997;95:885–91.
- [40] Basili S, Pignatelli P, Tanzilli G, et al. Anoxia-reoxygenation enhances platelet thromboxane A<sub>2</sub> production via reactive oxygen species-generated NOX2: effect in patients undergoing elective percutaneous coronary intervention. *Arterioscler Thromb Vasc Biol* 2011;31:1766–71.
- [41] Pignatelli P, Sanguigni V, Lenti L, et al. gp91phox-dependent expression of platelet CD40 ligand. *Circulation* 2004;110:1326–9.
- [42] Basili S, Pignatelli P, Basili S. Nutrition, supplements, and vitamins in platelet function and bleeding. *Circulation* 2010;121:1033–44.
- [43] Raad H, Paclet MH, Boussetta T, et al. Regulation of the phagocyte NADPH oxidase activity: phosphorylation of gp91phox/NOX2 by protein kinase C enhances its diaphorase activity and binding to Rac2, p67phox, and p47phox. *FASEB J* 2009;23:1011–22.
- [44] Barygina VV, Becatti M, Soldi G, et al. Altered redox status in the blood of psoriatic patients: involvement of NADPH oxidase and role of anti-TNF- $\alpha$  therapy. *Redox Rep* 2013;18:100–6.